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Compound Identification and Anticancer Activity of Ethyl Acetate Fraction from Bawang Sabrang (*Eleutherine palmifolia* (L.) Merr.) on HeLa Cervical Cancer Cell Line

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Abstract

Eleutherine palmifolia (L.) Merr. is a typical plant found in Central Kalimantan that has been used empirically by the Dayak people as medicine for various diseases, including cancer. The plant contains flavonoid compounds that potentially used as an anticancer. The purpose of this study is to find the most active fraction, indicated by its cytotoxic potency on HeLa cervical cancer cell line, and to identify compounds in *E. palmifolia* bulbs fraction. *E. palmifolia* bulbs was extracted by maceration. The extraction with ultrasonic bath and partition fractionation was conducted by using n-hexane, chloroform, and ethyl acetate. Each fraction was tested for toxicity level on HeLa cells using MTT assay. The identification of active compounds was carried out by Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). The result showed that based on the IC₅₀ value, the ethyl acetate fraction had the highest bioactivity. IC₅₀ values of n-hexane, chloroform, and ethyl acetate fractions were 250.77±19.01; 720.46±42.38; and 44.34±9.45µg/mL, respectively. The identification of the active compound in ethyl acetate fraction resulted 28 chemical compounds. Compounds with the highest percentage area were isoliquiritigenin and oxyresveratrol. The ethyl acetate fraction of *E. palmifolia* bulbs is potential to be developed as an anticancer candidate (phytopharmaceutical).

Keywords: Compound identification, Anticancer activity, *Eleutherine palmifolia* (L.) Merr., cervical cancer

INTRODUCTION

Cervical cancer is the second most common type of cancer among women after breast cancer and the third largest cause of death due to cancer in women. World Health Organization or WHO states that every year, more than 270,000 women died of

cervical cancer, and more than 85% of these deaths occur in developing countries. Indonesia is the sixth

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of 50 countries in the world with the highest deaths from cervical cancer which is 7,493 (WHO, 2013).

Eleutherine palmifolia (L.) Merr. ("bawang sabrang" in Indonesian) is a typical plant found in Central Kalimantan that has been used empirically by the Dayak people as medicine for various types of diseases such as cancer, hypertension, and diabetes. It can also be used to lower cholesterol, prevent ulcers as well as stroke, and reduce stomach pain after childbirth (Galingging, 2009). *E. palmifolia* bulbs contain bioactive compounds with anticancer effects. One of the bioactive compounds is flavonoid. Flavonoids are compounds proven to inhibit cancer cell proliferation (Mardiyarningsih, 2014). Inhibition of cervical cancer cells occurs in the path of estrogen/*Er-α* so that it can suppress cell proliferation on cervical cancer (Chung, *et al.*, 2010).

E. palmifolia has been discussed in many anticancer studies. The ethanol extract of *E. palmifolia* has a cytotoxic effect on HT29 colon carcinoma with LC_{50} of 3.125 mg/mL, and the p53 mutant can be suppressed by triterpenoid, flavonoid, anthraquinone, and coumarin compounds (Yusni, 2009). Ethanolic extract of *E. palmifolia* can inhibit the growth of cervical cancer HeLa cell with IC_{50} of 40.36 μ g/mL (Mutiah, *et al.*, 2017).

Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) is the development of LC-MS techniques which can be utilized to analyze compound. The method of chromatography can present reliable, high resolution of the chromatogram, accurate measurement of mass, and structural information as well as to detect a large number of metabolites in a sample of plants (Kumar, *et al.*, 2014). UPLC that is applied with MS is developed to be a powerful instrument to simultaneously identify and quantify chemical compounds contained in the raw materials of traditional medicines (Zao & Lin, 2014).

This study aims to determine the anticancer activity of *E. palmifolia* bulb fraction

on HeLa cervical cancer cell line and to analyze compounds of the most active fraction.

MATERIALS AND METHODS

Materials

The materials used in this study, *E. palmifolia* bulbs, were taken from Materia Medica Batu, Malang, Indonesia. Determination of the plant was done at the Indonesian Institute of Sciences with a reference number of 0065/IPH.6/HM/I/2017. HeLa cells were obtained from the collection of Parasitology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (UGM). Other materials were phosphate buffer saline (PBS) (Merck, Darmstadt, Germany), trypsin EDTA (Gibco, Invitrogen cell culture, Carisbad, USA), fetal bovine serum (FBS) (Gibco), penicillin-streptomycin (Gibco), Fungizone (Gibco), culture medium DMEM (Sigma-Aldrich, St Louis USA), DMSO (Sigma), 5 mg/mL MTT (Sigma) in PBS, 10% SDS in 0,1 N HCl (Merck), 96% ethanol (Sigma) aquadest, n-hexane (Sigma), chloroform (Sigma), and ethyl acetate (Sigma). The instrument used in this research were moisture analyzer (Mettler Toledo, Bekasi, Indonesia), a rotary evaporator (IKA, Ohio, USA), 96-well plate (Nunc, Denmark), ELISA reader (Bio-Rad, Jakarta, Indonesia), and Ultra Performance Liquid Chromatography-Quadrupole Time of Flight-Mass Spectrometry (UPLC-QtoF-MS) (Waters, Massachusetts, USA).

Extraction

E. palmifolia bulbs were washed clean, cut into small pieces, dried-puree, powdered using a blender and sifted. The powder was extracted using 96% ethanol solvent with ultrasonic bath for ± 18 minutes (min). The filtrate was then collected and evaporated using a rotary evaporator. Multilevel fractionation was then per-

formed using n-hexane, chloroform, and ethyl acetate solvent with a 1:1 solvent ratio. It was then concentrated and used as a test sample.

Cytotoxicity test using MTT assay

Cervical cancer HeLa cells were suspended in the culture medium (DMEM supplemented with FBS and penicillin-streptomycin) to reach 1.5×10^6 cells/mL. Cells (10^4 cells/100 μ L) were implanted into 96-well plates and then incubated for 24 h. A total of 1 mg of the fraction (n-hexane, chloroform, ethyl acetate) was dissolved in 100 μ L of dimethyl sulfoxide (DMSO). The solution was used as a stock. Furthermore, the fraction was made with a series of doses: 125; 62.5; 31.25; 15.625; 7.8125; and 3.90625 μ g/mL. Cisplatin concentration series that are 100; 50; 25; 12.5; 6.25; 3.125; and 1.5625 μ g/mL was used as positive control. After 24 h of incubation, the cells were observed and prepared for treatment using a test solution, which is n-hexane fraction, chloroform fraction, ethyl acetate fraction, and cisplatin. Each concentration was repeated in triplicate. Determination of anticancer activity was performed in vitro using MTT assay. MTT method is a colorimetric method based on the change of tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide] (MTT) into formazan in the mitochondria that are active in living cells (Doyle & Griffiths, 2000). The cells were there re-incubated for 24 h. At the end of incubation, 100 μ L of MTT reagent solution was added to the wells. The plate incubated for 4 hours to form formazan crystals. The number of formazan crystals formed correlates with cell viability. After 4 h incubation, the reaction of MTT was halted by the addition of stopper reagent sodium dodecyl sulfate (SDS) then the plate was incubated for another 24 h at room temperature. The measurement of absorbance was conducted using ELISA reader. Data obtained in the form of absorbance were subsequently converted to percentage of cell viability (Mutiah, 2014). The IC_{50} value was calculated using SPSS probit analysis.

Identification of active compound

Determination of active compound can be carried out by a chromatographic technique such as UPLC-QtoF-MS. This technique can be combined with bioassay cytotoxic test profiling and correlated with multivariate statistical analysis. This technique is faster and more efficient in the business discovery of new compounds (Wolfender, *et al.*, 2015). The active compounds of the most-active fraction were identified using ACQUITY UPLC HSS C18 column in tandem with MS tandem quadrupole system, ESI positive ion mode. The mobile phase was a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The source temperature was 100°C, and the desolvation temperature was 350°C. A 10 mg extract sample was solved in 10 mL volumetric flask with absolute methanol then 5 μ L volume was injected into the UPLC-MS system. From chromatogram data, the area was presented in percentage. The chromatogram was processed using Masslynx version 4.1 software (Waters, Massachusetts, USA). The component identification was based on the ratio of measured m/z in Masslynx and ChemDraw version 12.0 (CambridgeSoft, Cambridge, USA).

RESULTS

Cytotoxicity test

Extracts were tested for the in vitro anticancer activity on HeLa cell line. The IC_{50} values were 250.77 ± 19.01 ; 720.46 ± 42.38 , and 44.34 ± 9.45 μ g/mL for n-hexane, chloroform, and ethyl acetate fractions, respectively. The most active fraction was ethyl acetate. Table 1 showed the IC_{50} values from each fraction.

Identification of active compounds

The compound of ethyl acetate fraction was analyzed using UPLC-MS. Identification of the active compound was done using MassLynx software version 4.1 and compared to a database (ChemSpi-

der, ChemDraw). The results of the chromatogram sample are shown in Figure 1.

Each chromatogram peak indicated one compound. The application of Masslynx 4.1 was used to process the chromatogram to find out the m/z spectrum. Therefore, the molecule formula of the interpretation product compound could predict. Then, the ChemSpider website helped the researcher to find out the compound name of the prediction. By integrating and linking compounds from hundred of data sources, the whole molecule should be taken away by one atom. After finding the name of the compound and structure, the measured and calculated m/z were compared by drawing the compound structure using ChemDraw Ultra 12.0

(Skoog, *et al.*, 2004). If the difference is ≤ 0.0005 , then the peak belonged to the predicted compound (Brenton & Godfrey, 2010). From data interpretation, 38 compounds were revealed that consisted of 19 known structural formula and 9 unknown structures. The obtained active compounds, molecular formulas, and compound structures are shown in Table 2. The data interpretation showed that 2 compounds had the highest concentration in the sample, which are isoliquiritigenin (retention time of 8.629; structural formula of $C_{15}H_{11}O_4$) and oxyresveratrol (retention time of 6.291; structural formula of $C_{14}H_{12}O_4$). The mass spectra and the structural formula of those two compounds is shown in Figures 2 and 3.

Table 1. IC_{50} Values of Each Fraction

Fraction	Average of % viability HeLa cells ($\mu\text{g/mL}$) \pm SD*						$IC_{50} \pm SD^*$ ($\mu\text{g/mL}$)
	125	62.5 3	1.25 1	5.63 7	.81	3.91	
N-hexane	30.29 \pm 3.40 1	6.07 \pm 0.72	5.78 \pm 1.59	2.31 \pm 0.69	28.90 \pm 1.06 8	4.05 \pm 5.25	250.77 \pm 19.01
Chloroform	37.57 \pm 5.56 1	8.61 \pm 3.67	18.26 \pm 3.34 1	4.45 \pm 0.60	15.72 \pm 1.64 1	8.27 \pm 2.11	720.46 \pm 42.38
Ethyl acetate	32.95 \pm 2.69 2	9.83 \pm 9.65	51.79 \pm 8.33 8	0.12 \pm 4.34	99.92 \pm 0.13 9	7.61 \pm 4.14	44.34 \pm 9.45

*Average \pm standard deviation, triplicate for each experiment

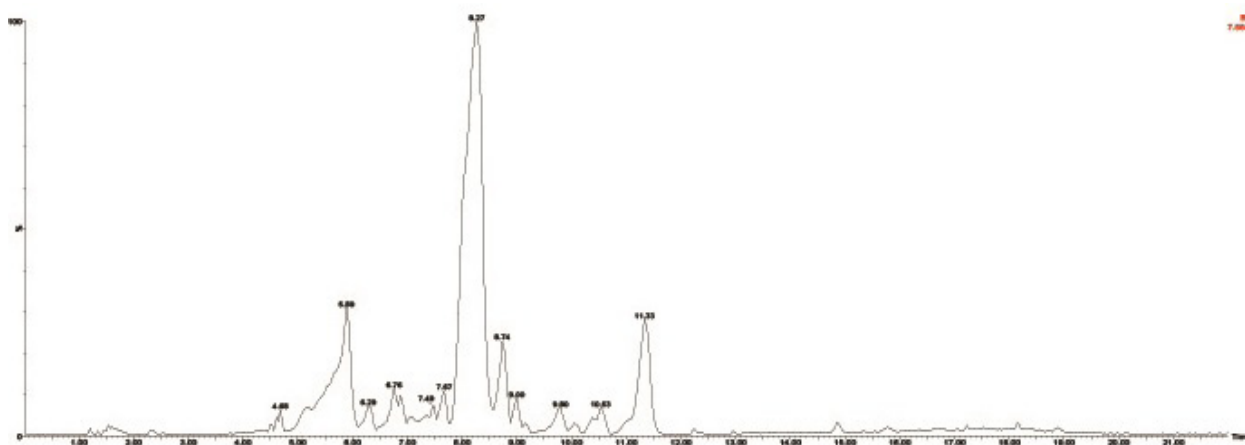
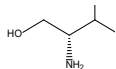
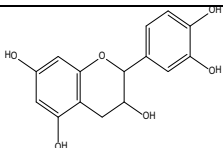
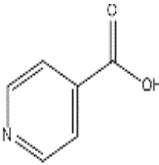
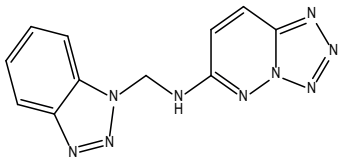
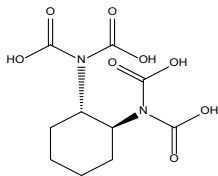
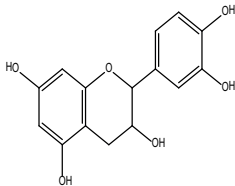
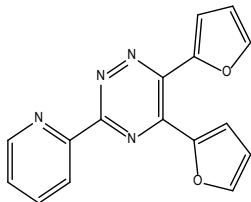
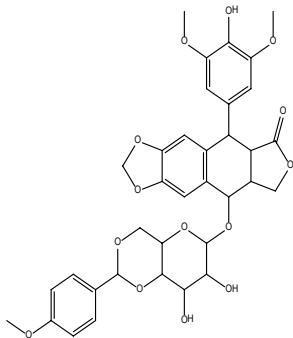
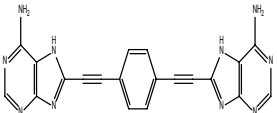
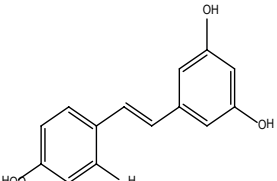
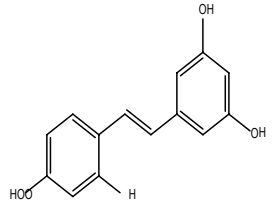
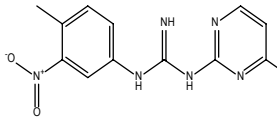


Figure 1. UPLC-MS chromatogram of Ethyl Acetate Fraction from *Eleutherine palmifolia* (L.) Merr. Bulb

Table 2. The Active Compounds and Molecular Formula

Rt (min)	Measure M/Z	Calculated M/Z	% Area	Formula	Structural Formula
				Compounds Name	
1.352	103.0997	103.0997	0.0544	C ₅ H ₁₃ NO L-(+)-Valinol	
1.535	290.0791	290.079	0.6879	C ₁₅ H ₁₄ O ₆ D-(+)-Catechin	
2.301	123.0321	123.032	0.1567	C ₆ H ₅ NO ₂ Isonicotinic acid	
2.552	267.2495	267.2495	0.0471	C ₁₁ H ₉ N ₉ N-(1H-Benzotriazol-1-ylmethyl)tetrazolo[1,5-b]pyridazin-6-amine	
3.764	290.075	290.075	0.0082	C ₁₀ H ₁₄ N ₂ O ₈ N,N'-(1S,2S)-1,2-Cyclohexanediylbis(imidodicarbonic acid)	
4.382	290.0791	290.079	0.1417	C ₁₅ H ₁₄ O ₆ D-(+)-Catechin	
4.679	290.0804	290.0804	0.8771	C ₁₆ H ₁₀ N ₄ O ₂ 3-(2-Pyridyl)-5,6-bis(2-furyl)-1,2,4-triazine	
5.891	419.1355	-	14.6482	C ₂₂ H ₁₈ N ₄ O ₅ UNKNOWN	UNKNOWN

Rt (min)	Measure M/Z	Calculated M/Z	% Area	Formula Compounds Name	Structural Formula
8.738	680.2105 6	80.2105	5.2187	$C_{35}H_{36}O_{14}$ Furo[3',4':6,7]naphtho[2,3-d]-1,3-dioxol-6(5aH)-one, 5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)-9-[[4,6-O-[(4-methoxyphenyl)methylene]hexopyranosyl]oxy]-	
9.001	725.2082 -	1	.7712	$C_{36}H_{36}O_{16}$ UNKNOWN	UNKNOWN
9.435	392.1247 3	92.1246	0.0881	$C_{20}H_{12}N_{10}$ 8,8'-[Benzene-1,4-dyl-di(ethyne-2,1-diyl)]diadenine	
9.801	244.0736 2	44.0736	1.7966	$C_{14}H_{12}O_4$ Oxyresveratrol / 4-[(E)-2-(3,5-Dihydroxyphenyl)vinyl]-1,3-benzenediol	
10.053 2	59.0984	-	0.4578	$C_{16}H_{10}N_4$ UNKNOWN	UNKNOWN
10.533 3	59.0767	-	2.0586	$C_{18}H_{14}O_8$ UNKNOWN	UNKNOWN
11.333 2	44.0736	244.0736 9	.9249	$C_{14}H_{12}O_4$ Oxyresveratrol / 4-[(E)-2-(3,5-Dihydroxyphenyl)vinyl]-1,3-benzenediol	
14.855 3	00.1335	300.1335 0	.484	$C_{14}H_{16}N_6O_2$ 1-(4,6-dimethylpyrimidin-2-yl)-3-(4-methyl-3-nitrophenyl)guanidine	

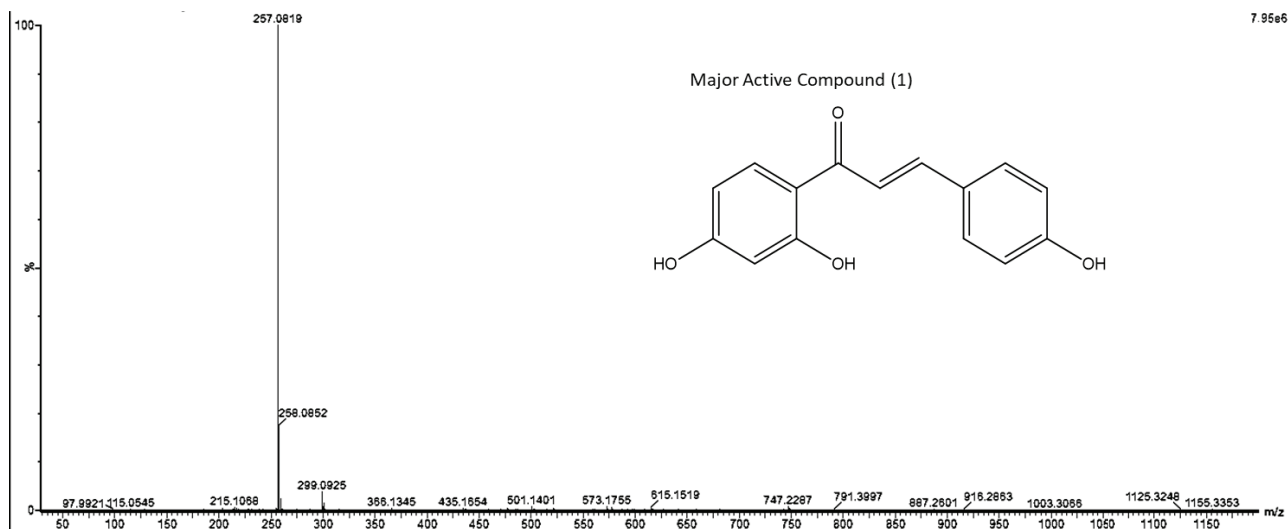


Figure 2. Mass Spectra and Structure Formula of Isoliquiritigenin

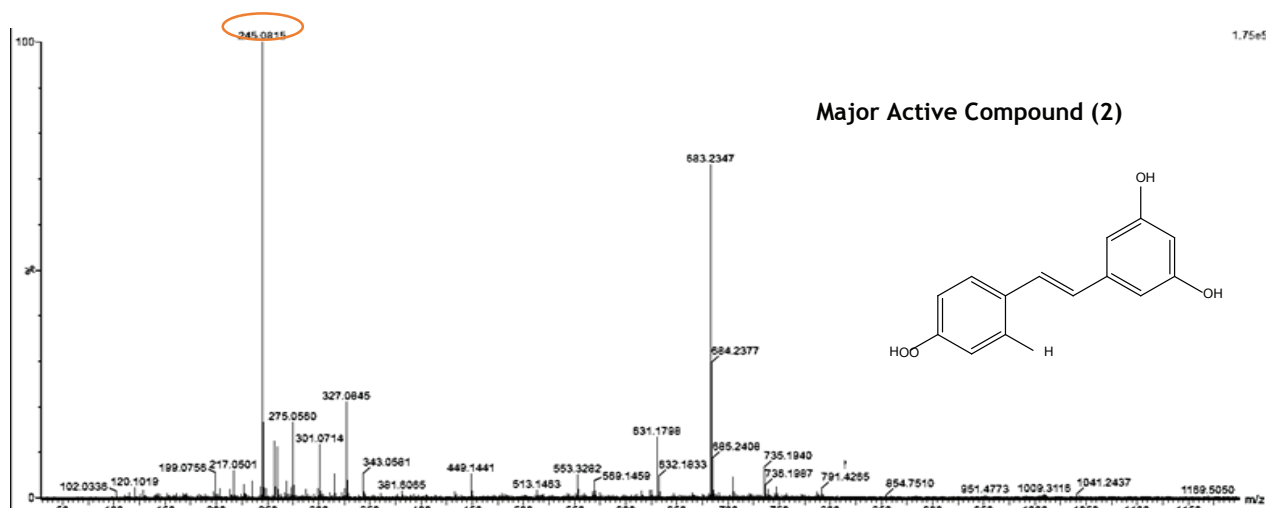


Figure 3. Mass Spectra and Structure Formula of Oxyresveratrol

DISCUSSION

This study aims to determine the cytotoxic of onion extracts and fractions of *E. palmifolia* and to determine the active compounds, which has the highest anticancer activity. The cytotoxic level of the sample fraction against cervical cancer cell was stated with IC_{50} value that indicates the amount of concentration producing 50% cell proliferation

resistance. The obtained IC_{50} values showed that the ethyl acetate fraction had the lowest value at $44.34 \pm 9.45 \mu\text{g/mL}$ (Table 1). The IC_{50} value of an extract obtained under $100 \mu\text{g/mL}$ indicates their potential to be used as a chemopreventive agent (Suzery, et al., 2014). The ethyl acetate fraction can thus be further developed as a chemopreventive agent. Based on the provisions of National Cancer Institute, an extract declared active has anticancer

activity if it has a value of $IC_{50} < 30 \mu\text{g/mL}$, moderately active if $30 \mu\text{g/mL} \leq IC_{50} < 100 \mu\text{g/mL}$, and inactive if $IC_{50} > 100 \mu\text{g/mL}$ (Rahmawati, *et al.*, 2013). Based on the results, it can be seen that the ethyl acetate fraction of *E. palmifolia* bulb is in moderately active category, while the n-hexane sample fraction and chloroform of *E. palmifolia* bulbs are said to be inactive or do not have anticancer activity. Cisplatin is also used as a positive control with an IC_{50} of $19.77 \mu\text{g/mL}$.

As a result of the cytotoxic activity test the ethyl acetate fraction has the highest potential cytotoxicity. Two major compounds were obtained and suspected as lead compounds of anticancer activity of *E. palmifolia* bulbs. The major active compound with the retention time of 8.269 min is known as isoliquiritigenin (1). Several previous studies have shown that isoliquiritigenin compounds of the flavonoid group have anticancer activity against B16 melanoma cells (Iwashita, *et al.*, 2000), colon cancer cells (Wang, *et al.*, 2013), breast cancer cells (Hou, *et al.*, 2017), and squamous cells (Takahashi, *et al.*, 2004). Isoliquiritigenin triggered mitochondrial and endoplasmic reticulum stress to induced apoptosis in HeLa cell. The antiproliferative activity of isoliquiritigenin against cervical cancer by decreased cell viability, induced cell accumulation in G2/M phase and morphological and biochemical features of apoptosis. Isoliquiritigenin was reported inhibit NF- κ B activity. NF- κ B is constitutively activated in human cancer, including cervical cancer (Hirchaud, *et al.*, 2013). The major active compound with a retention time of 11.333 min is known to be an oxyresveratrol (2). Previous research proved that the derivative of oxyresveratrol, 3',5'-Diacetoxy-2,4-diisopropoxystilbene, is selectively cytotoxic against cervical cancer HeLa cells (Chatsumpun, *et al.*, 2016).

CONCLUSION

Ethyl acetate fraction has the highest bioactivity toward HeLa cervical cancer cell line with

IC_{50} of $44.34 \pm 9.45 \mu\text{g/mL}$. The identification of active compounds showed that the major compounds contained in the ethyl acetate fraction of *E. palmifolia* bulbs are isoliquiritigenin and oxyresveratrol. The compounds were suspected to be responsible for the anticancer activity of *E. palmifolia* bulbs.

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